

1994

## The Physiological, Enzymatic, and Genetic Characterization of *Staphylococcus* sp. Chromium (VI) Reductase Function

Matthew J. Trudeau  
Colby College

Follow this and additional works at: <https://digitalcommons.colby.edu/seniorscholars>

 Part of the [Biology Commons](#)

Colby College theses are protected by copyright. They may be viewed or downloaded from this site for the purposes of research and scholarship. Reproduction or distribution for commercial purposes is prohibited without written permission of the author.

---

### Recommended Citation

Trudeau, Matthew J., "The Physiological, Enzymatic, and Genetic Characterization of *Staphylococcus* sp. Chromium (VI) Reductase Function" (1994). *Senior Scholar Papers*. Paper 374.

<https://digitalcommons.colby.edu/seniorscholars/374>

This Senior Scholars Paper (Open Access) is brought to you for free and open access by the Student Research at Digital Commons @ Colby. It has been accepted for inclusion in Senior Scholar Papers by an authorized administrator of Digital Commons @ Colby.

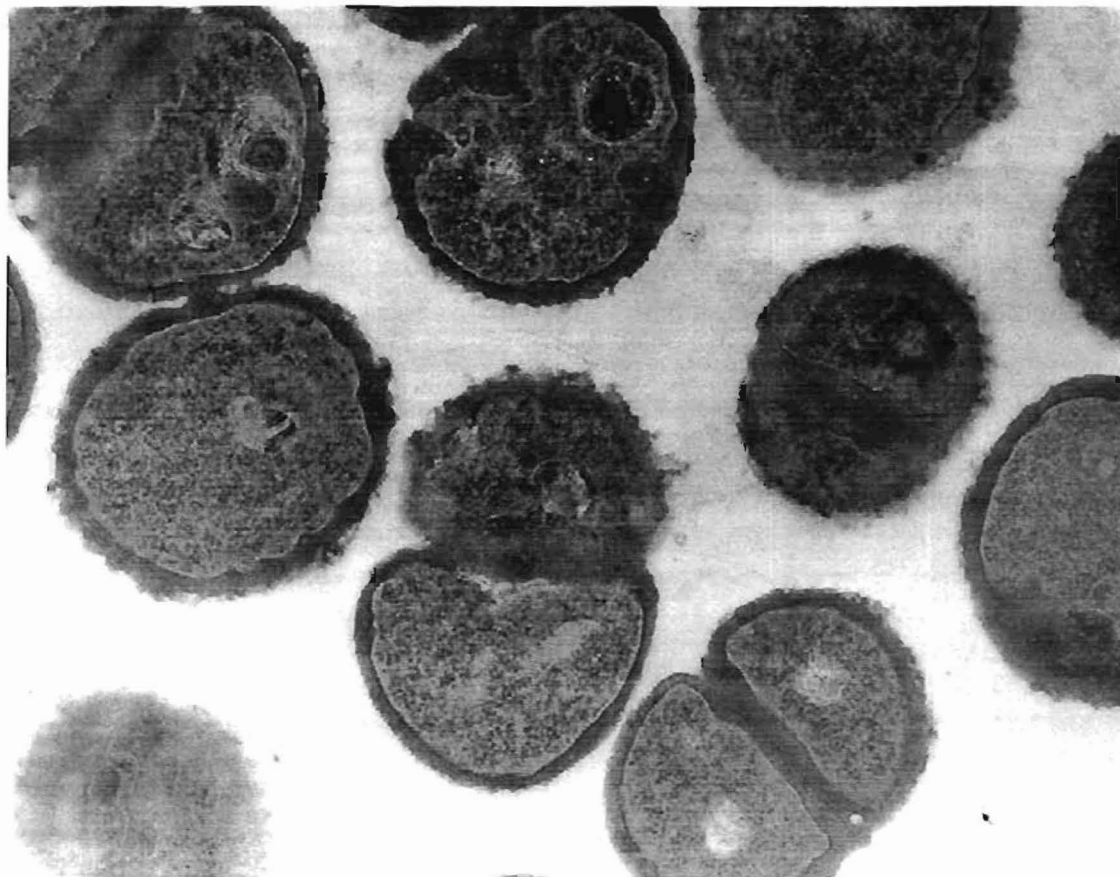
**THE PHYSIOLOGICAL, ENZYMATIC, AND GENETIC  
CHARACTERIZATION OF *Staphylococcus* sp.  
CHROMIUM(VI) REDUCTASE FUNCTION**

Matthew J. Trudeau

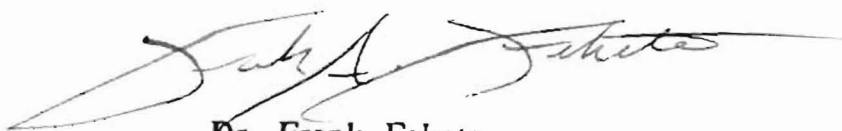
Conducted Under the Advisement of  
Dr. Frank A. Fekete

Submitted in Partial Fulfillment of the Requirements of the  
Senior Scholar's Program

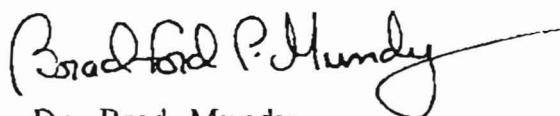
**COLBY COLLEGE**  
1994



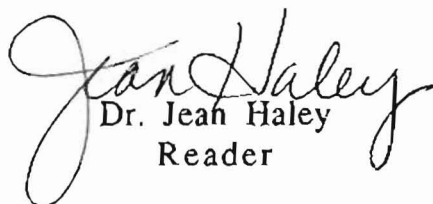
PPROVED



Dr. Frank Fekete  
Senior Scholar Tutor



Dr. Brad Mundy  
Chair, Department of Chemistry



Dr. Jean Haley  
Reader



Dr. Fernando Gouvea  
Chair, Senior Scholar Committee

## *TABLE OF CONTENTS*

Acknowledgments	ii
Abstract	iii
Introduction	1
Materials and Methods	
Cell Culture Maintenance	5
Growth and Viability Quantitation	9
Bacterial Isolate Identification	9
Reduction Assay	10
Protein Analysis	12
Genetic Characterization	15
Results	
Microbiological Analysis	20
Protein Analysis	31
Genetic Analysis	35
Discussion	38
Bibliography	48



## ACKNOWLEDGMENTS

A number of individuals lent their time and wisdom to me throughout the course of this project and I am indebted to them. Dr. Paul Greenwood's instruction on Fluorescence Spectroscopy proved valuable for cell lysis analysis. Dr. Jean Haley helped me to look at the problem of genetic characterization from a multitude of angles. Dr. Dave Bourgaize's instruction was instrumental in guiding me through protein and genetic analysis. I am also grateful to Holly Carmichael who provided guidance relative to plasmid isolation technique. David Berner's electron micrograph appears on the cover of this report. Thanks to all of these people for their help.

I also thank Dr. Frank Fekete for his guidance both in and out of the laboratory. He was a motivating force who helped, more than anyone else, to drive this project as far as it got. Thank you Frank for your time and patience.

Finally I would like to thank Dr. Brad Mundy, Dr. Haley and Dr. Fekete again for laboring through and editing the early drafts of this work.

All funding for this project was derived from Colby College grants.

## ABSTRACT

A strain of *Staphylococcus* isolated by Dr. Fekete at the Sandia National Laboratory toxic metal dumping site in Sandia, New Mexico, has been found to reduce toxic Cr(VI) to the less toxic Cr(III) state. We have ascertained the environmental parameters for optimal bacterial growth and Cr(VI) reduction. This knowledge may be employed in a comprehensive bioremediation scheme designed to accelerate natural reparation of that Sandia ecosystem.

In addition we have investigated the genetic and enzymatic basis for this Cr(VI) reducing ability. This information may allow us to create more effective bioremediation schemes based on the comprehensive knowledge of enzyme and gene function. Preliminary investigations have been carried out toward this end which may serve as the basis for a more thorough investigation.

## INTRODUCTION

The United States military industrial complex laid waste to large parcels of land in the American Southwest during the explosive arms race following the second World War. Astonishing quantities of heavy metals associated with weapons research were dumped onto the desertscape. Toxic chromium(VI) was one of these metals. As a result, this land is inhospitable to most life. However, certain resilient forms of bacterial life, like this *Staphylococcus* isolated by Dr. Fekete (unpublished) at the Sandia National Laboratory in New Mexico have been found not only to withstand high chromium(VI) levels but also to enzymatically reduce the metal to the less toxic Cr(III) state.

Aside from military weapons research, chromate compounds have many industrial applications and often are the cause of serious environmental degradation in marine and freshwater sediments. Cr(III) and Cr(VI) enter the environment as a result of effluent discharge from steelworks, electroplating, tanning industries, oxidative dying, chemical industries, cooling towers, as well as defense-related industries. The metal may also enter drinking water systems from the corrosion inhibitors used in water pipes or containers or by contamination of the ground water from sanitary landfill leaching (13). Hexavalent chromium compounds are water soluble, toxic, and highly carcinogenic (11). Therefore, it is of major concern to understand the nature of chromium in natural systems which have been polluted by industrial effluents.

During the 1930's chromium toxicity was observed in American chromate miners who experienced a mortality rate from lung cancer 300% greater than expected based on the contemporary national mortality data (11). Since then, simple hexavalent chromium salts have been demonstrated to induce point mutations in spot tests in which Cr(VI) is dropped onto plates pre-inoculated with *E. coli* (6). Cr(VI) has also been found to induce chromosomal aberrations in human lymphocyte cultures, hamster embryonic cells, a Chinese hamster cell line, and in mouse carcinoma cells lines (11).

Chromate species fall into the transition-causing class of mutagens causing point mutations during DNA replication. Chromium compounds are known to decrease the fidelity of DNA synthesis *in vitro*. It is highly likely that they specifically cause G-C to A-T transitions during DNA synthesis (11).

While nucleotide transitions may be important for mutation induction, a more immediate cause which allows for the ability of Cr(VI) to induce chromosomal aberrations may be that chromium competes with calcium for DNA binding sites. Calcium, believed to be essential for the structural integrity of chromosomes, shows less binding to RNA in the presence of Cr(VI). DNA has a lower affinity for calcium than does RNA, and should therefore be more affected by chromium (11).

The interest in chromium is governed, in part, by the fact that its toxicity critically depends upon its oxidation state. While Cr(III) is considered an essential cofactor for mammalian maintenance of glucose, lipid, and protein metabolism, Cr(VI) is known to be toxic to

most life forms (13). Trivalent chromium is much less soluble and approximately 100 times less toxic than hexavalent Chromium (10).

An uptake-reduction model accounts for the differing toxicities of Cr(VI) and Cr(III) compounds. According to Jenette's model (11), the greater toxicity exhibited by Cr(VI) is the result of its more efficient uptake into cells and its ability to undergo subsequent intracellular reduction. Cr(III), which does not normally reach high concentrations in the cell, is then capable of reacting directly with cellular proteins and nucleic acids. Direct oxidation of certain critical molecules by intracellular Cr(VI) may also occur. Cr(VI) salts represent a serious biological hazard. Thus, reduction from Cr(VI) to Cr(III) represents a potentially useful detoxification process.

The *Staphylococcus* isolate of study may be manipulated to do just that. Bioremediation is a revolutionary technique in which biological catalysts are used to detoxify the polluted site (10). An ability to enhance the Cr(VI) reducing capability of this bacterium yields a potentially effective bioremediation strategy for reparation of the desert ecosystem from which the bacteria were isolated.

Using an array of integrated protein and genetic analysis techniques, as well as a comprehensive microbiological analysis, considerable data were derived from this isolate relative to its reductase activity. Whole-cell enzyme reduction activity analysis, substrate preference analysis, optimal environmental parameter analysis, partial isolation and subsequent study of the reductase enzyme, and preliminary genetic characterization provide key insights to the reduction mechanism employed by the organism.

In addition to the possible whole-cell bioremediation applications, there is also the potential that purified native reductase enzyme may be dispersed into the affected environment encapsulated in an inert carrier, or used in a fixed bed bioreactor. Under the proper conditions (such as pH, temperature, and presence of specific coenzymes), the enzyme could be allowed to reduce chromium before denaturing under environmental stress. This methodology would avoid the dangers associated with introducing nonindigenous organisms to these toxic dumps.

Similarly, genetic cloning may allow us to "improve upon nature." Transformation of the reductase gene into another bacterium, which thrives to a greater extent under a given set of environmental parameters, could potentially create an organism that would accomplish the given remediation more efficiently. Dangers of bioaugmentation associated with introducing genetically reorganized life into an ecosystem could be minimized by tight regulation. Certainly, there are numerous potential bioremediation schemes which may be derived from an integral understanding of this Cr(VI) reducing *Staphylococcus sp.* isolate

An understanding of the Cr(VI) reducing capacity, from bacterial nutrition requirements to gene function, is essential for an integral knowledge of the cell's physiological and biochemical resistance to Cr(VI) toxicity, and that is the objective of this project. This understanding may help in creating an effective bioremediation scheme for Sandia. In addition, a comprehensive understanding of this Cr(VI) function may allow for a generalized Cr(VI) detoxification procedure, applicable irrespective of toxic site location.

Knowledge of the reductase function may not only be used for construction of a bioremediation scheme and protein engineering applications, but it will also contribute to a greater understanding of Cr(VI) reductases found in a variety of genera. Many bacteria have been found to have the ability to enzymatically reduce Cr(VI) to Cr(III) (3,5,9,10,12,14,15,17,19,25), and it has been assumed that a similar process occurred in this *Staphylococcus* isolate. Clearly, the proven ability to manipulate this reductase function carries with it a myriad of important potential environmental applications.

## MATERIALS AND METHODS

### I. Cell Culture Maintenance

#### A. Growth and Storage Procedure

##### (1) Media Preparation

Bacteria were initially grown and assayed for reductase activity on m Plate Count Broth (Difco). Having determined growth rates relative to enzyme activity on this complex media, it was then necessary to determine activity on defined salts media. As the composition of Plate Count Broth is not completely understood it is compulsory to ascertain positive reductase conditions in the presence of a defined minimal media in order to understand the physiological parameters governing this bacterial metabolism and reductase capability. This medium is composed of various components including salts, trace elements, vitamins, and carbon sources

necessary for bacterial growth. By substituting various carbon sources we ascertained the preferable carbon source for growth and enzyme activity.

The minimal media recipe was adapted from Fekete et al. (9):

Solution I: Salts

$K_2HPO_4$	14.0g
$KH_2PO_4$	6.0g
Na citrate	1.0g
$MgSO_4 \cdot 7H_2O$	0.2g
$(NH_4)_2SO_4$	2.0g
<hr/>	
100 ml $H_2O$	autoclave sterilization

Solution II: Trace Elements

Fe Citrate	0.3 g
$MnSO_4 \cdot H_2O$	0.002g
$H_3BO_3$	0.001g
$CuSO_4 \cdot 5H_2O$	0.001g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.002g
$ZnSO_4$	0.001g
EDTA	0.05g
$CaCl_2 \cdot 2H_2O$	0.02g
<hr/>	
100 ml $H_2O$	autoclave sterilization

Solution III: Vitamin Solution

Pyridoxine-HCl	0.2g
Niacinamide	0.2g
Nicotin Acid	0.2g
Thiamine-HCl	0.4g
Biotin	0.008g
<hr/>	
1L $H_2O$	filter sterilization



### Solution IV: Carbon Source

### 20% Sugar Solution

autoclave sterilization

Alternative Sugars: 20% solutions

## Glucose

## Sucrose

Citrate

Amino Acid Supplement: 30% solution

autoclave sterilization

Casamino acids (Sigma)

### Buffering and pH:

PIPES buffer (Sigma)

 $\text{NaOH}$ 

Agar:

## Lab Grade Agar

## Toxic Metal

$K_2CrO_4$  (Sigma)

autoclave sterilization

Minimal stock media was prepared separately to avoid caramelization of sugars in the presence of salts at high temperature. In one 250 ml Erlenmeyer flask, (1) 43 ml of sterile dH<sub>2</sub>O, (2) 5 ml salt solution, and (3) 1.5 g PIPES buffer were combined and adjusted to pH 7.0 with NaOH. In a second Erlenmeyer flask, (1) 50 ml sterile reversed osmosed deionized purified H<sub>2</sub>O (dH<sub>2</sub>O), (2) 1 ml 20% sugar solution, (3) 1.5 g Agar (this ingredient was deleted in liquid media preparation), and (4) 1 ml of Casamino acid solution (where required) were combined. The volume was adjusted with 1 ml sterile dH<sub>2</sub>O in the absence of casamino acids.

The two mixtures were autoclaved and then combined. Complete minimal media requisites were completed with the addition of 750  $\mu$ l of vitamin solution. Where appropriate, toxic

Cr(VI) was added at varying concentration depending upon experimental conditions.

The agar media was poured into culture plates (approximately 25 ml per plate). The nutrient broth as well as agar plates were stored at 4°C until use.

## 2. Inoculation

Plates were inoculated and bacteria were transferred on plating media according to standard aseptic technique. Inoculated plates were incubated at 37°C.

Similarly, 3 ml liquid media were inoculated from plate cultures and grown over night at room temperature. The following day the 3 ml culture was diluted to 103 ml and the temperature raised to 37°C. This pre-inoculation scheme was employed to ensure sufficient cell density for growth and tolerance studies.

### **B. Long Term Culture Preservation**

In order to safeguard against contamination, loss of culture, spontaneous failure to thrive, or genetic alteration over time, *Staphylococcus sp.* cultures were deep-frozen at -80°C for preservation.

Cells were grown up overnight at 37°C to maximum growth. In 2 ml Corning cryogenic tubes, 0.25 ml sterile glycerol, which prevents the formation of ice crystals, was added to 0.8 ml liquid culture. The solution were then frozen at -80°C. This method should provide for colony integrity for at least one year (8).

## II. Growth and Viability Quantitation

Bacterial growth in liquid media was monitored with respect to increase in absorbance at 540nm relative to a baseline reading of liquid media with no cells. All readings were made periodically with a Spectronic 20 (Milton Roy Company).

In order that increases in absorbance could be correlated directly with population size a viable plate count was carried out concurrently with a growth curve. At every interval of spectroscopic analysis, 50.0  $\mu$ l aliquots were withdrawn from the culture and diluted serially, from  $10^{-3}$  to  $10^{-8}$ . From each of these dilutions 100  $\mu$ l were plated using the spread plate technique onto complex media plates. The culture plates were incubated overnight at 37°C and colonies were counted. From these data a curve was constructed relating cell viability to absorbance. This method allows for differentiation of an increase in cell population, from an accumulation of dead cells. The approximate viable population size may be correlated to absorbance.

## III. Bacterial Isolate Identification

Bacterial isolates were sent to a commercial laboratory for identification. The laboratory, Analytical Systems in Essex Junction, Vermont, employed fatty acid composition analysis as a means of determining species. Fatty acid profiles are species specific.

#### IV. Reduction Assay:

In order to assay for Cr(VI) reduction, a diphenylcarbazide assay was employed (23). Diphenylcarbazide is a ligand which binds to Cr(VI) and emits purple light. The decrease in purple light intensity as a result of bacterial Cr(VI) reduction activity was measured over time at 540 nm (Spectronic 20).

##### 1. Assay Solution and Standard Curve Construction

The diphenylcarbazide solution was prepared in the following manner: 0.25g S-diphenylcarbazide (Sigma) was combined with 40g phthalic anhydride (Sigma) in 100 ml of 95% ethanol. The solution was stored at 4°C.

A standard curve was constructed so that any reduction in absorbance could be correlated to a specific decrease in Cr(VI) concentration. Figure 1 illustrates the Cr(VI) concentration standard curve. A standard reference point of zero chromium and a consistent time interval (approximately 10 minutes) between sampling and reading was done to minimize reading variation.

For each assay point, 0.8 ml diphenylcarbazide reagent was added to 0.25 ml of Cr(VI) solutions of varying concentration in a 1.0 ml quartz cuvette and read with a Beckman DU-70 spectrophotometer.

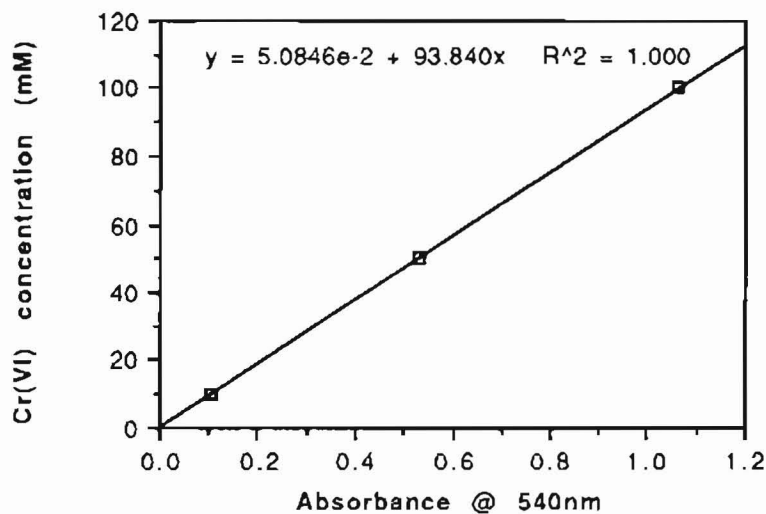


Figure 1. Cr(VI) concentration standard curve using diphenylcarbazide assay.

## 2. Reduction Activity Assay

Cells were grown up overnight at 37°C in 100 ml Plate Count broth. The cells were centrifuged at 5000xg for 5 minutes and resuspended in 10 ml TE buffer (10 mM Tris HCl pH=8, 2 mM EDTA). 0.2 ml 1 mM Cr(VI) was added to the mixture to give a final 20  $\mu$ M Cr(VI) concentration. The solution was immediately assayed using the diphenylcarbazide protocol to give a relative zero point. 0.4 ml of cell suspension was centrifuged at 5000xg for 5 minutes. Then, 0.25 ml of the supernatant was added to the diphenylcarbazide reagent as described above. The solution was placed on a rotary shaker at 200 rpm at 30°C and sampled every 10-15 minutes.

## 3. Reductase assay in the presence of coenzymes

In order to prove reductase enzyme activity and disprove non-specific Cr(VI) cell binding, coenzyme tests were run. Horitsu and

Ishibashi (13,14) have reported coenzyme enhancement of reductase activity particularly in the presence of NADH and NADPH.

Cells were centrifuged and resuspended as described above. NADH was added to the reaction mixture to a final concentration of 10 $\mu$ M. Cr(VI) reductase activity was monitored as described above over time intervals of 5-10 minutes.

## V. Protein Analysis

### A. Cell Lysis

Enzyme denaturation results in a loss of activity. Therefore, in order to study the native Cr(VI) reductase enzyme the bacterial cells had to be disrupted in such a way as to maintain native protein conformation. A number of cell disruption protocols were employed to no avail including sonication, lysozyme, and freeze-thaw methodology. Finally, a protocol employing enzymatic lysis using lysostaphin (Sigma) proved effective. Lysostaphin is an enzyme found by Tuncan and Martin (22) to be specific to *S. aureus*.

#### 1. Lysostaphin Lysis Protocol

*Staphylococcus sp.* were grown overnight in 150 ml complex media. The cells were centrifuged at 5000xg for 5 minutes and washed twice in 100 ml Hank's Tris Buffered Salts (HTBS) (Sigma). Cells were then centrifuged again at 5000xg for 5 minutes and resuspended in 40 ml HTBS. 510 Units lysostaphin (Sigma) were added to the solution. The solution was allowed to sit at room temperature for 40 minutes(20). The suspension was transferred to 25 ml Corex tubes

and centrifuged at 21,000xg for 1 hour. The pelleted membrane fraction was separated from the soluble fraction.

The membrane pellet was resuspended in 40 ml HTBS. Both the membrane fraction and soluble fraction were assayed for activity as described above in the presence of 20 $\mu$ M Cr(VI). Also, identical tests were run in the presence of 10 $\mu$ M NADH or NADPH.

## B. Cell Lysis Assay

In order to verify cell lysis prior to protein purification and characterization, and enzyme kinetics studies, a variety of assays were carried out.

### 1. Biorad Total Protein Detection System

The Biorad protein assay consists of a protein binding agent which absorbs at 595nm in a protein-bound state. Based on a standard curve, established with buffered egg white lysozyme, a correlation exists between the absorbance of the reaction solution and the protein concentration in that solution. Cell lysis was confirmed by an increase in extracellular protein. Figure 2 illustrates the standard curve established so that this correlation could be employed.

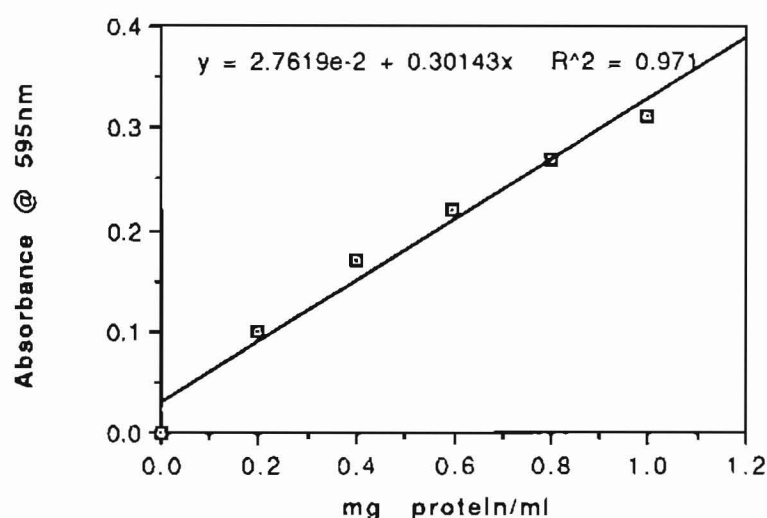


Figure 2. Standard protein concentration curve for Biorad total protein assay.

Cells were treated by various lysis procedures as described above. The suspension was assayed with the Biorad system before and after the lysis procedure. Reaction mixtures were centrifuged at 5000xg for 30 minutes. 4 ml Biorad reagent was assayed with 100  $\mu$ l reaction solution. After a 5 minute incubation at room temperature samples were observed spectrophotometrically. An increase in protein levels, relative to the pre-lysis value, in the supernatant would indicate total or partial cell lysis.

## 2. Hoechst Staining

Hoechst staining, a slightly more complex but far more definitive assay for cell lysis, was employed after the Biorad data proved inconclusive. Hoechst stain is a fluorescent dye which binds tightly to DNA. Intact cells will absorb ultraviolet light, because they contain DNA. However, if the cells are Lysol, and their contents



dispersed, the extracellular environment will absorb ultraviolet light with equal or greater intensity.

Cell suspension (10  $\mu$ l) was air-dried on a microscope slide and washed with ethanol. After drying, the slide was treated with 10  $\mu$ l Hoechst stain, covered with a glass slip and viewed with a fluorescence microscope (Zeiss).

### 3. Assay for Lysostaphin Activity

When lysostaphin is used for cell lysis, Hoechst stain is ineffective because the Hoechst stain binds to the lysostaphin and produces a high background. Another lysis assay was necessary. As stipulated by Sigma, one unit of enzyme should reduce the turbidity( $A_{620}$ ) of a suspension of *S. aureus* cells from 0.250 to 0.125 in 10 minutes at pH 7.5 at 37°C in a 6.0 ml reaction mixture. Although our isolate was not found to be a *S. aureus*, these guidelines sufficed for monitoring positive lysostaphin activity on our *Staphylococcus* isolate.

## **VI. Genetic Characterization**

### **A. Plasmid Curing**

Elimination of a plasmid from a bacterial culture is the best way to substantiate or refute that a genetic trait is carried on a plasmid. A phenotype linked to the presence of a plasmid will not be expressed in cured cultures (12).

Using a mutagen such as acridine orange the bacterium may be cured of its plasmids (accessory DNA which is separate from the genome) under the proper conditions. A series of 12 ml tubes

containing varying concentration of acridine orange (10 µg/ml-250 µg/ml) in complex media (pH 7.6) were inoculated with  $10^3$ - $10^4$  cells (interpolated from viability curve) and incubated overnight at 37°C. The culture which showed just a slight increase in turbidity was plated out in serial dilutions ( $10^{-3}$ - $10^{-7}$ ) on 1 mM Cr(VI) plates. If cured of their plasmids, and assuming the reductase enzyme is encoded on plasmid DNA, bacteria will not grow on these high level Cr(VI) plates.

## B. Plasmid Isolation

Assuming that the Cr(VI) reductase function is encoded on a plasmid, as many resistance and accessory genes are (2,5), it was important to detect any bacterial plasmids and characterize them. Localization of the reductase gene to a plasmid would allow us the potential to clone the gene and genetically modify another bacterium, thereby confirming Cr(VI) reductase activity.

### 1. Plasmid Isolation Protocol

Cells were grown overnight in 4 ml Plate Count media. The suspension was then centrifuged at 5000xg for 5 minutes and washed with 2 ml TE buffer. The washed pellet was resuspended in 40 µl TE buffer.

The cells were then lysed by the addition of 600 µl freshly made lysis solution (0.5% SDS in TE, pH=12.4). The suspension was incubated at 37°C for 20 minutes with gentle agitation. Then, 450 µl acetate solution (60.0 ml 5M potassium acetate, 11.5 ml glacial acetic acid, brought up to 100 ml with sterile dH<sub>2</sub>O) was added to the

solution which was then held at 4°C for 5 minutes. Centrifugation at 15000xg for 20 minutes at 4°C allowed for separation of the membrane fraction.

The supernatant was transferred to a sterile Eppendorf tube. A phenol:chloroform extraction was performed to precipitate extraneous protein. The mixture was shaken gently and centrifuged at 15000xg for 2 minutes at 4°C. The aqueous phase (upper layer) was removed, being careful to avoid the protein interface, to a sterile Eppendorf tube. To this phase 800 µl 95% ethanol was added to precipitate the plasmid DNA. This solution was left at room temperature for 5 minutes and microfuged at 15000xg for 5 minutes.

The supernatant was aspirated and the pellet was rinsed quickly with 100 µl 70% ethanol to remove any protein residue. The solution was spun at 15000xg briefly and the 70% ethanol aspirated. The pellet was allowed to air-dry and was resuspended in 25 µl TE buffer.

## 2. Cell Lysis Assay

In order to assure that cell lysis had occurred to a significant extent before plasmid extraction the Hoechst stain was used exactly as it was described in the cell lysis assay in preparation for protein purification.

## 3. Plasmid Visualization

Aliquots of the *Staphylococcus* sp. plasmid isolation solutions were separated by electrophoresis on 0.8% agarose TBE gels (0.8 g agarose, 80 ml dH<sub>2</sub>O, 20.0 ml 5X TBE (Sigma), 5 µl ethidium

bromide). A positive control *Escherichia coli* plasmid extraction aliquot known to contain three plasmids and Lambda phage (digested with EcoRI restriction endonuclease) molecular weight standards were also separated on the gel.

### C. Establishment of Genomic Library

In order to attain a copy of every gene present in the genome, a genomic library is established by which the genome is isolated and completely cloned in fragments. I have been able to isolate the genome but have not cloned the genome before the printing of this thesis.

#### 1. Genomic Isolation

Genomic isolation procedure was adapted from Dyer et al. (7). Cells were grown overnight in 100 ml Plate Count media broth. The suspension was centrifuged at 5000xg for 5 minutes and the cell pellet was washed twice in 10.0 ml TES (30 mM Tris-HCl pH=8, 50 mM NaCl, 5 mM EDTA). The washed pellet was resuspended in 10 ml TES (2.5 M NaCl) and transferred to a 250 ml Erlenmeyer flask.

340 units lysostaphin were added and the suspension was put in a rotary shaker at 200 rpm for 1 hour at 37°C. Sarcosyl was added to a final concentration of 2%. Guanidine-HCl crystals were added to a final concentration of 7.5 M. This solution was allowed to shake gently for 1.5 hours at 55°C after which it was diluted with dH<sub>2</sub>O to 5.8 M guanidine-HCl.

This solution was run through a 2 step cesium chloride gradient (2.9 ml 2.8 M CsCl/2.0 ml 5.7 M CsCl, prepared in 20 mM Tris/20 mM

EDTA, pH=8, 100  $\mu$ l ethidium bromide. This step gradient was centrifuged at 108,000xg for 22 hours at 20°C.

A DNA band typical of this protocol was not obtained. Instead, a DNA-protein aggregate floating in the CsCl was removed with a wide mouthed pipet to avoid shearing the genomic DNA. The solution was dialyzed 3 times against 3L TE buffer for 4 hours each. The DNA was precipitated with an equal volume of isopropanol. The DNA was spooled from the solution with a hooked pasteur pipette, resuspended in TE buffer, and allowed to sit overnight to allow for complete solubilization of the DNA. A phenol:chloroform extraction was performed to remove protein. The aqueous layer was transferred to a sterile tube with care to avoid the protein interface. 0.6 volumes of 2-propanol and 0.1 volume of Na-acetate were added to again precipitate the DNA from solution. The solution was held at 4°C for 30 minutes and then centrifuged at 22,000xg for 20 minutes at 4°C. The supernatant was discarded and the pellet dried. After drying the DNA pellet was resuspended in 1.5 ml TE buffer and allowed to solubilize overnight.

## 2. Cell Lysis Assay

Cell lysis for genomic isolation was assayed by the lysostaphin lysis assay as described under the protein analysis section.

## 3. Genome Visualization

Aliquots of isolated genomic DNA were separated by electrophoresis through a 0.8% agarose gel as described above. The

aliquot was run with control Lambda phage DNA (digested with Eco R1) to determine the size of genomic fragments. DNA fragments of at least 15 Kb are required for cloning(26).

## RESULTS

### I. Microbiological Analysis

#### 1. Cr(VI) Dose Dependent Inhibition of Growth

Bacteria were found to survive on Cr(VI) levels up to 3.0 mM on Plate Count agar plates. As expected, decreasing viability of bacterial populations corresponded to increases in Cr(VI) concentration. This relationship was best quantitatively represented in liquid Plate Count culture, where increasing culture turbidity can be monitored spectrophotometrically with respect to bacterial growth. Figures 3 and 4 illustrate this observation.

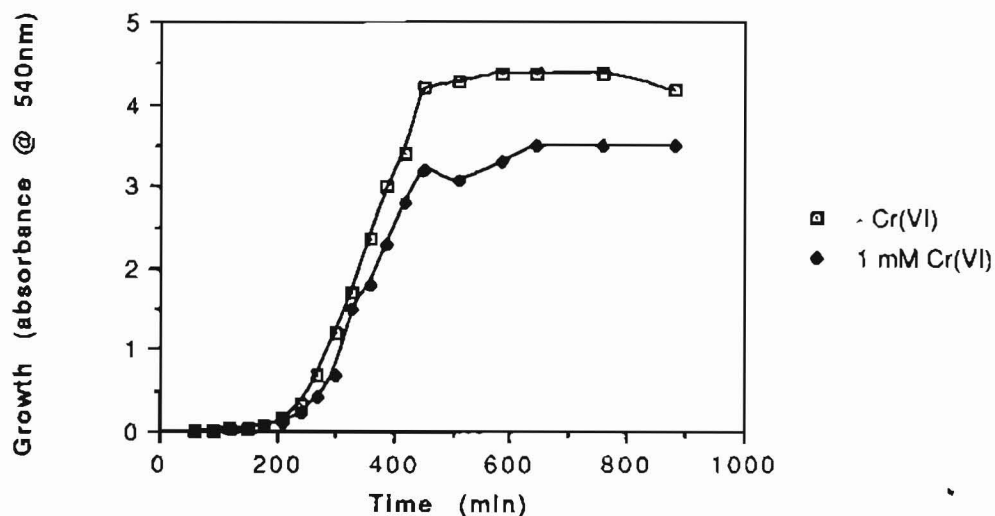


Figure 3. *Staphylococcus* sp. growth in the absence and presence of 1.0 mM Cr(VI). Cells were grown in plate count liquid media and agitated in a rotary shaker at 37°C.

There was an observed 22% difference in growth between cells grown in the presence of 1 mM Cr(VI) and cells grown in the absence of Cr(VI). Similarly there was the observed dose dependent inhibition of growth corresponding to increasing Cr(VI) levels, as is shown in figure 4.

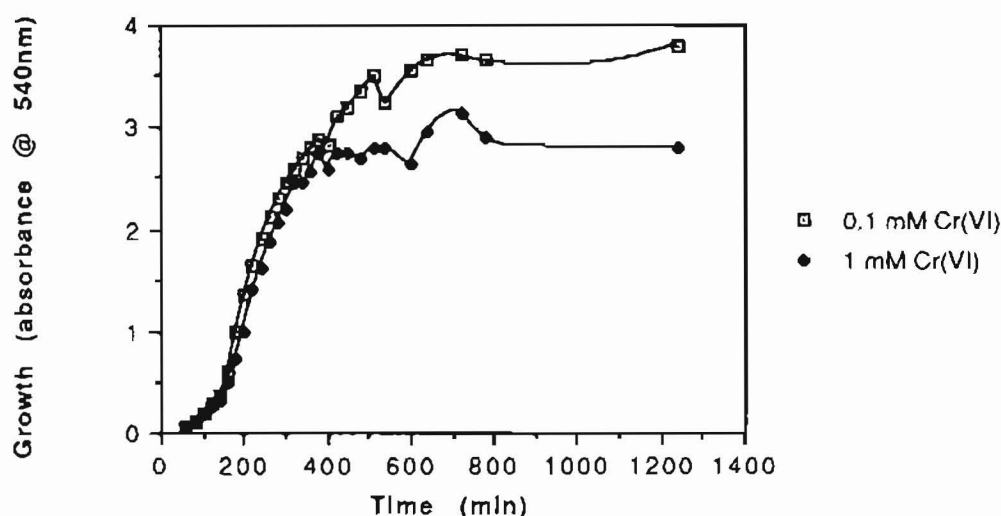


Figure 4. *Staphylococcus* sp. growth as a function of Cr(VI) concentration. Cells were grown in plate count liquid media and agitated in a rotary shaker at 37°C.

### Viability Assay

A viability assay was run so that absorbance levels corresponding to cellular growth could be correlated directly to viable cell population, thereby taking account of cell debris and other absorbing material resulting from cell death. These data proved essential to many techniques, including inoculation of assay cultures. Growth rates in the presence of chromium did not adhere to rates of changing absorbance. Log phase growth, which appears to be similar by spectrophotometric analysis, are revealed to be quite dissimilar

by this method. With these data, inoculated cultures could be initially equilibrated based upon the derived cell count relative to a given absorbance.

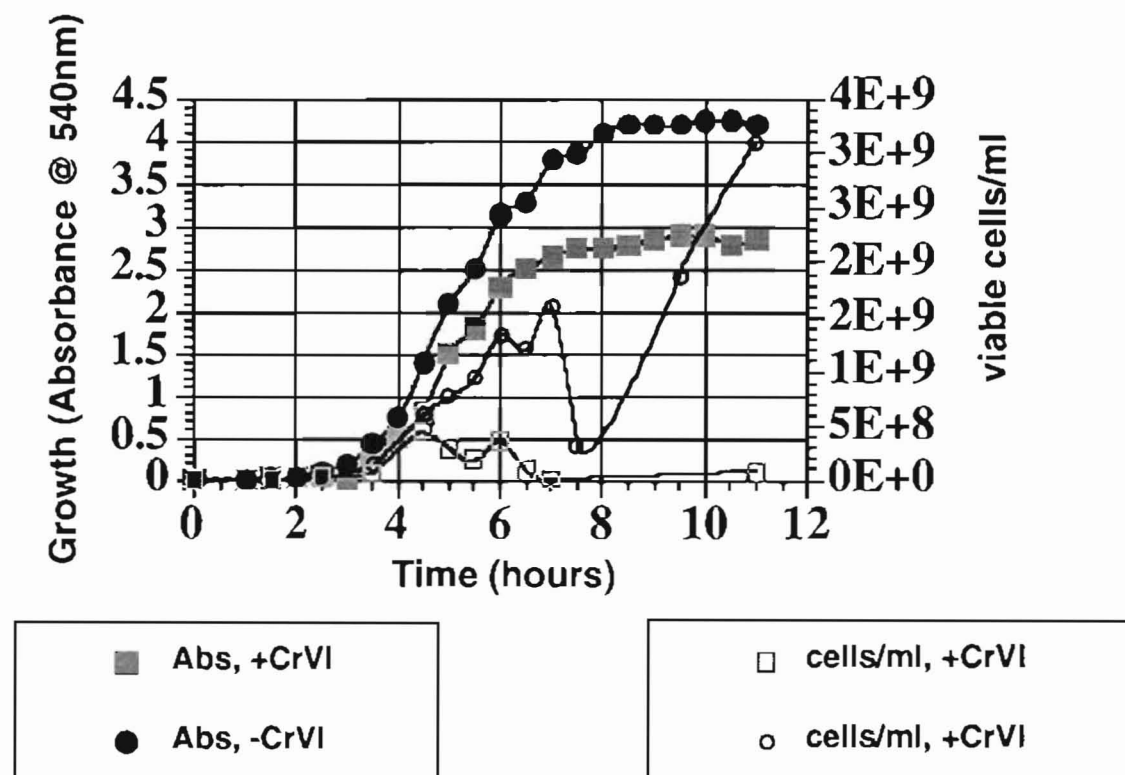


Figure 5. Viability Curve of *Staphylococcus* isolate in the absence and presence of Cr(VI) with respect to growth quantified by spectrophotometric analysis (540nm). Cells were grown in plate count liquid media and agitated in a rotary shaker at 37°C, and sampled over time.

### C. Reductase Activity

In addition to possessing the capability of tolerating high concentrations of chromium, the organism was also observed, with the diphenylcarbazide assay, to have the ability to reduce levels of toxic Cr(VI). Figure 6 illustrates the organism's ability to efficiently reduce Cr(VI).



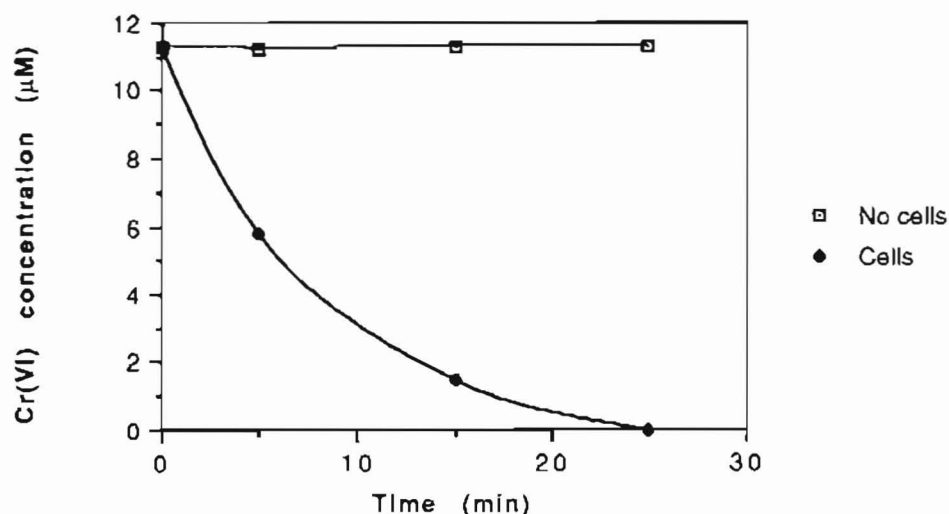


Figure 6. *Staphylococcus* Reduction Capabilities. Cells were introduced to 10  $\mu\text{M}$  Cr(VI) solution and observed for reduction against 10  $\mu\text{M}$  Cr(VI) solution in the absence of cells.

#### D. Bioremediation Parameters

Having demonstrated significant bacterial Cr(VI) reduction, the optimal physiological conditions under which the isolate reduces Cr(VI) were established. These parameters include optimal pH, temperature and carbon source.

The following figures illustrate optimal pH for growth of this strain as viewed by spectrophotometric analysis. This approach assumed that population size and viability corresponded directly to reductase activity. Figures 7 and 8, together, reveal the high and low end of higher bacterial pH tolerance and also display the optimal pH range for cell proliferation.

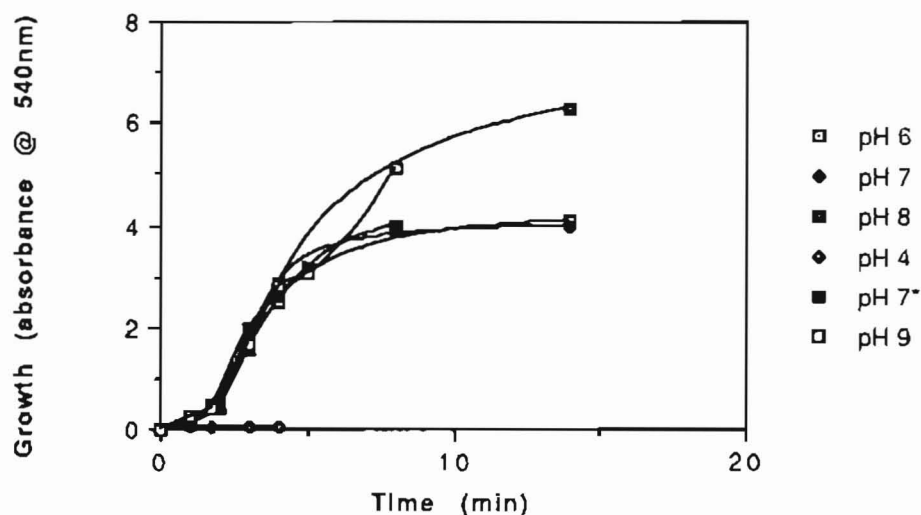


Figure 7. Bacterial growth at pH 4 to 9 was monitored spectrophotometrically with respect to time. Cells were grown in plate count liquid media at 37°C and agitated at 200 rpm in a rotary shaker. \*=duplicate pH assay repeated to ensure a consistent reading frame.

Figure 7 establishes the lower limit for growth at pH 6, and the optimal pH between 8 and 9. A second set of data, Figure 8, establishes the upper limits for growth at pH 11 and verified that the optimal pH for cellular growth and reductase activity was indeed between 8 and 9.

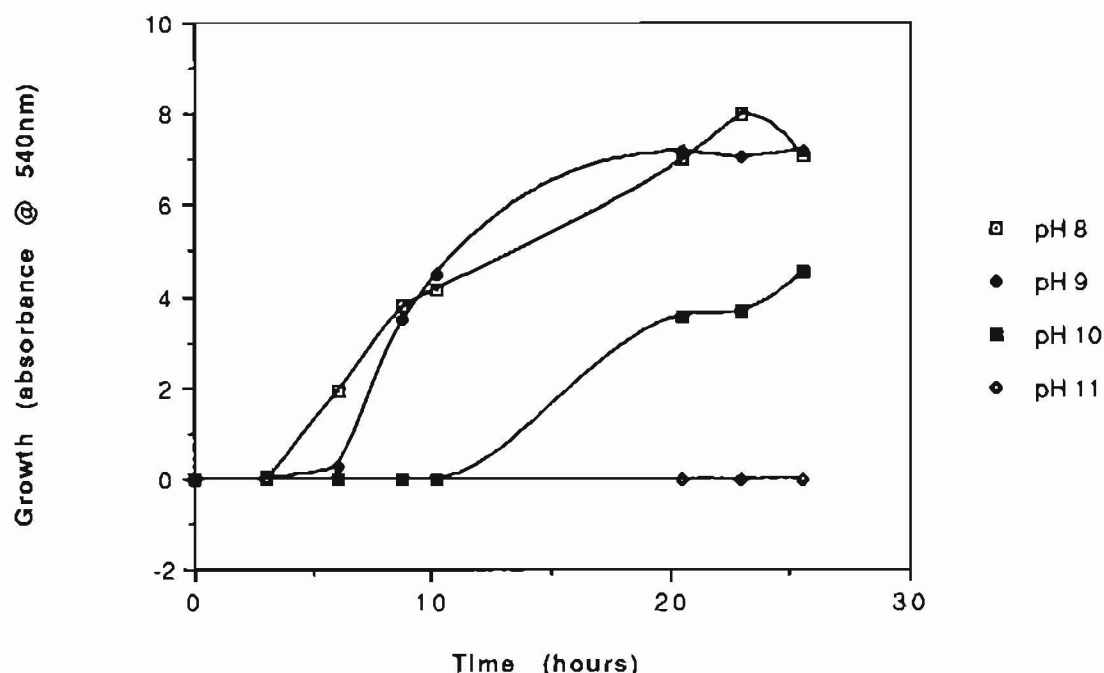


Figure 8. Bacterial growth at pH 8 to 11 was monitored spectrophotometrically with respect to time. Cells were grown in plate count liquid media at 37°C and agitated at 200 rpm in a rotary shaker.

Having ascertained the optimal pH for Cr(VI) reduction on the assumption that optimal Cr(VI) reduction corresponded to optimal cell growth yielded unfounded data. Figure 9 illustrates that optimal growth did indeed coincide with optimal Cr(VI) reduction. Thus, the aforementioned assumption and the data derived from this line of experimentation was verified.

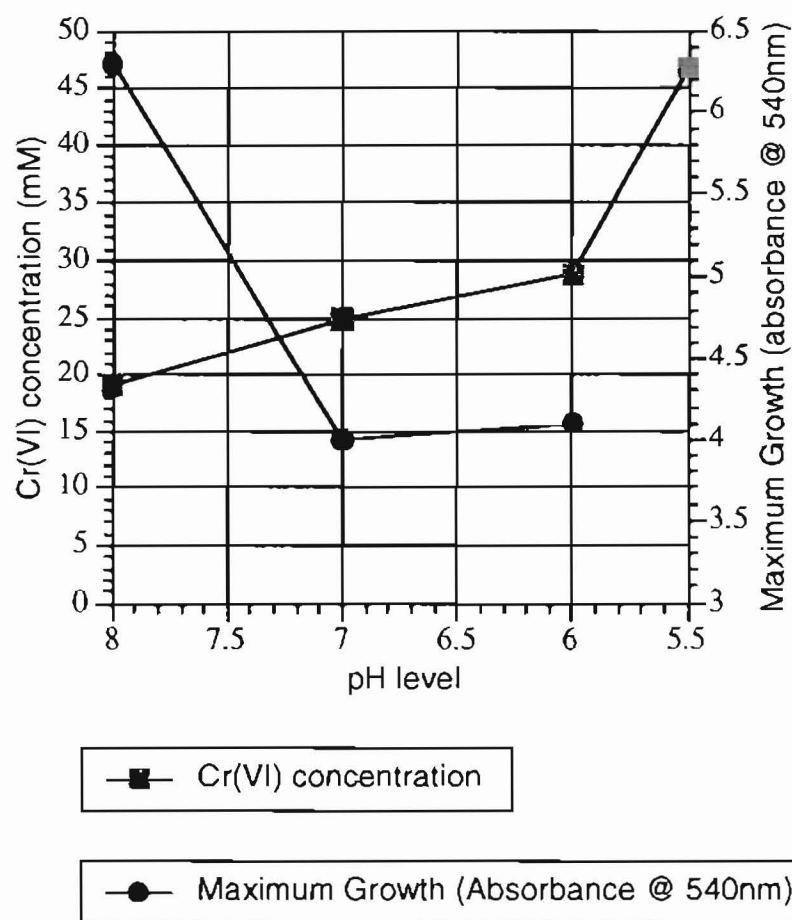


Figure 9. Whole cell Cr(VI) reductase activity vs. growth was observed at various pH levels.

Figures, 10, 11, and 12, reveal the optimal incubation temperature relative to growth monitored by spectrophotometric analysis. As each temperature study was done under variant conditions, the data could not be compiled onto one figure. Bacteria grew most efficiently at  $\sim 37^{\circ}\text{C}$ .

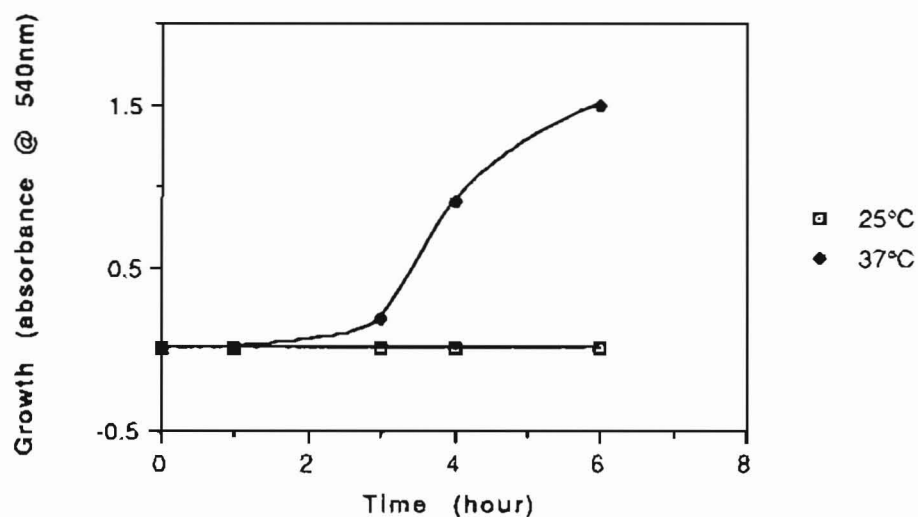


Figure 10. Growth rates were monitored at 25°C and 37°C incubation temperatures in plate count media and agitated at 200 rpm in a rotary shaker.

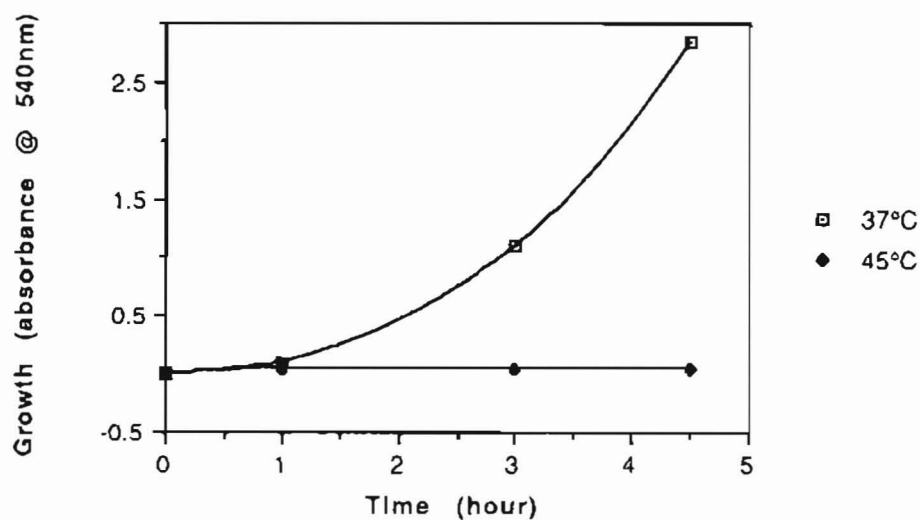


Figure 11. Growth rates were monitored at 37°C and 45°C incubation temperatures in plate count media and agitated at 200 rpm in rotary shaker.

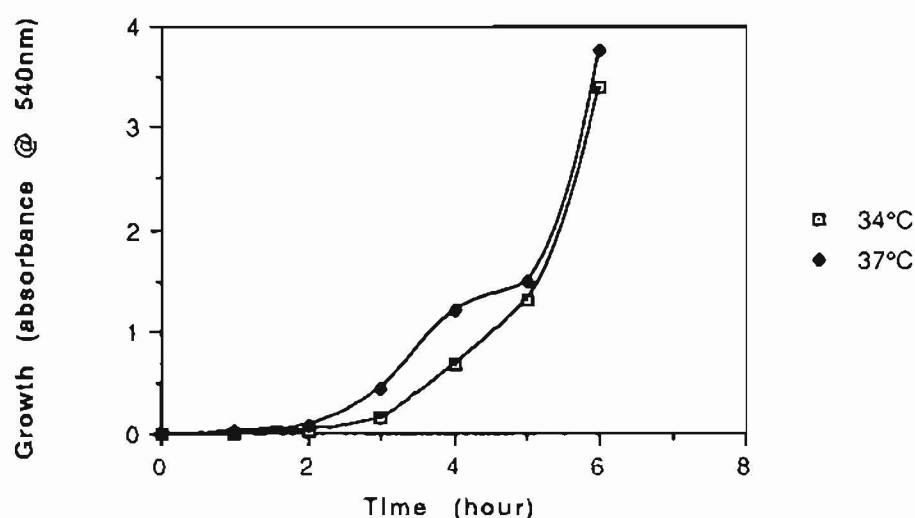


Figure 12. Growth rates were monitored at 34°C and 37°C incubation temperatures in plate count media and agitated at 200 rpm in a rotary shaker.

As stated above, a comprehensive understanding of Cr(VI) reduction must include determination of the optimal carbon source for bacterial growth as it corresponds to reduction capabilities. Data revealed that *Staphylococcus* sp. may indeed grow on a variety of substrates including sucrose and citrate. However, both of these substrates showed sparse bacterial growth relative to Plate Count media. As figure 13 illustrates, Cr(VI) tolerance capabilities of the cells were markedly decreased as well, when grown with the various carbon source starvation conditions. When supplemented with casamino acids, the bacteria grew at an accelerated rate, relative to the identical media without casamino acid supplement, and were better able to acclimate to the toxic environment.

These data were observed on plate culture and growth was observed after two days. Growth levels were visually examined and assigned relative values. As expected, the cells grew better in the presence of a Plate Count medium which provided almost all of the

requisite cell nutrients and took from the cell the burden of synthesizing many cellular biomolecules. Also as anticipated, Cr(VI) tolerance capabilities were at optimal levels in this nutrient rich media.

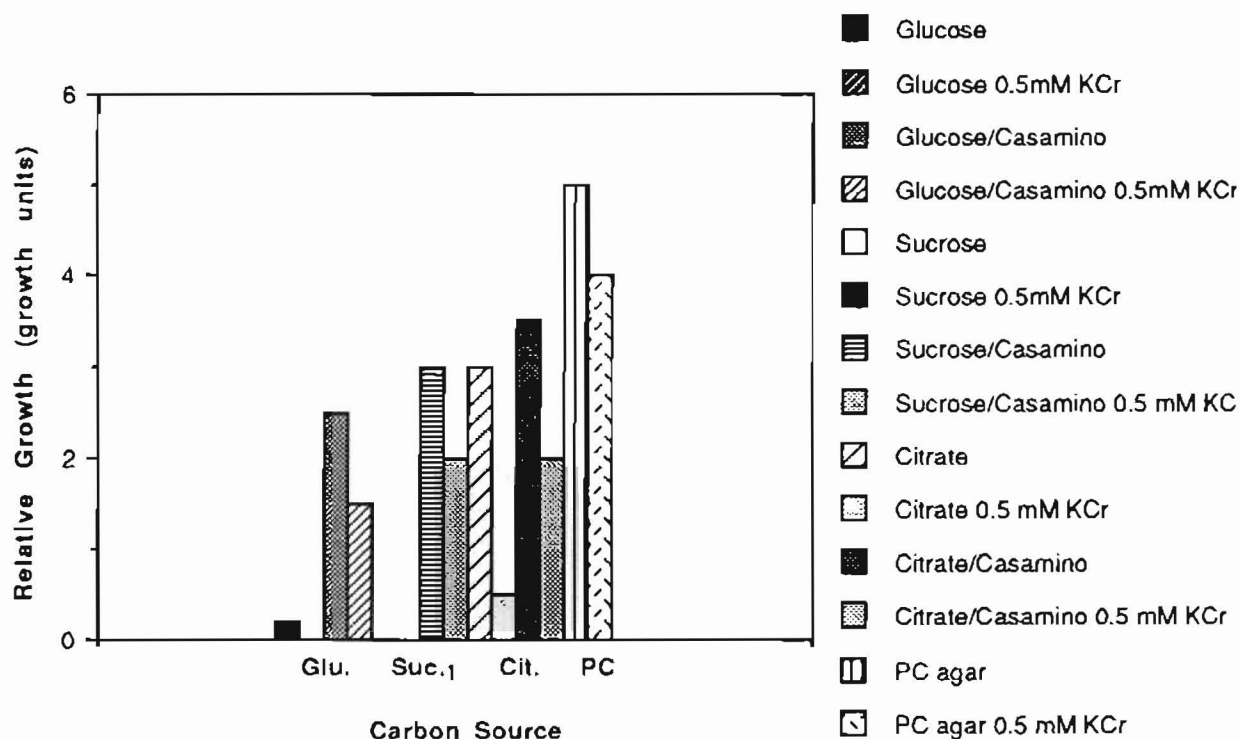


Figure 13. Relative growth in the presence of various oxidizable substrates. Growth was viewed on plate culture and assigned relative growth units.

#### D. Co-enzyme Reductase Activity Assay

Previous studies have reported a Cr(VI) reductase coenzyme dependence (14,15,16,21). In order to understand enzyme activity optima and kinetics of this organism, and also to show that an enzyme was indeed responsible for reduction, a coenzyme activity assay was performed. Coenzymes provided the enzyme with the necessary electrons for Cr(VI) reduction catalysis, and yielded higher Cr(VI) reduction rates. NADH was introduced to whole cell cultures

which were then assayed for reductase activity. Figure 14 illustrates the bacterial dependence upon this coenzyme. In the presence of 100  $\mu\text{M}$  NADH, Cr(VI) reduction occurred at a much faster rate.

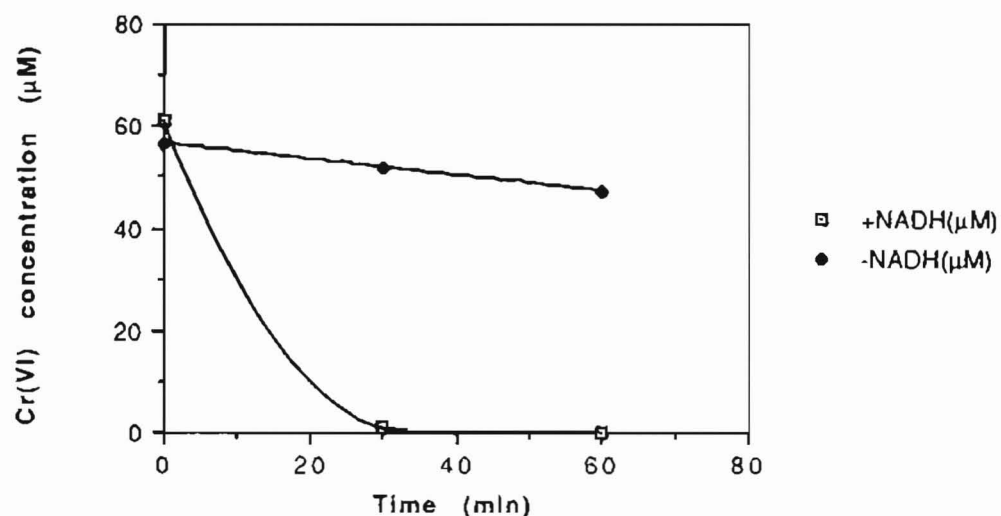


Figure 14. Whole cell Cr(VI) reduction as a function of NADH. Cells were assayed in the presence and absence of 10  $\mu\text{M}$  NADH for Cr(VI) reduction.

The assay was run again in the presence of  $\text{NAD}^+$  and its reduced form, NADH. Reduction initially occurred approximately 90% faster in the presence of NADH with respect to that in the presence of  $\text{NAD}^+$  or in the absence of coenzyme.



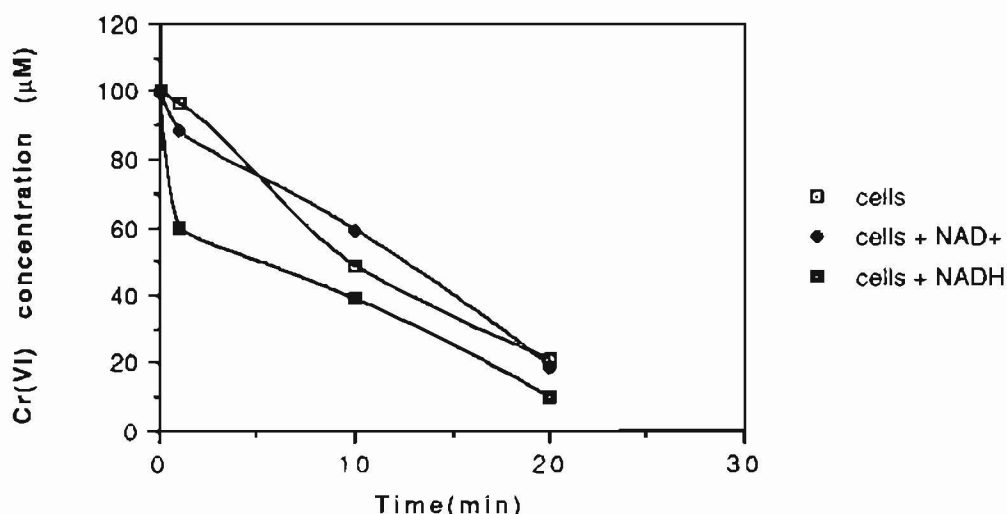


Figure 15. Whole cell Cr(VI) reduction as a function of NADH and NAD<sup>+</sup>. Cells were assayed for Cr(VI) reduction in the presence of 10 μM NADH and 10 μM NAD<sup>+</sup>.

#### 4. Identification

Bacterial isolates were identified by Analytical Systems Inc, Essex Junction, Vermont. They determined, based on fatty acid analysis, that the isolate's fatty acid composition resembled that of *Staphylococcus carnosus*. Statistical analysis provided by Analytical Systems yielded a positive identification value of 0.387. A value above 0.4 indicates a very good match to the microbial identification database, while a value of 0.25 or lower indicates a poor match. Thus, 0.387 yields an inconclusive isolate identification as *S. carnosus*.

## II. PROTEIN ANALYSIS

### 1. Identification of Reductase Activity in Cell Fractions

Using the lysostaphin disruption technique, soluble and membrane fractions of the *Staphylococcus sp.* were isolated and

assayed for activity. These fractions were assayed in the presence of NADPH which was subsequently found (section II.2) to be the major reducing agent employed by Cr(VI) cellular reductase function. The reductase activity was localized to the soluble fraction of the cell (Figure 16). Cell lysis was confirmed by the lysostaphin lysis assay and the Biorad assay.

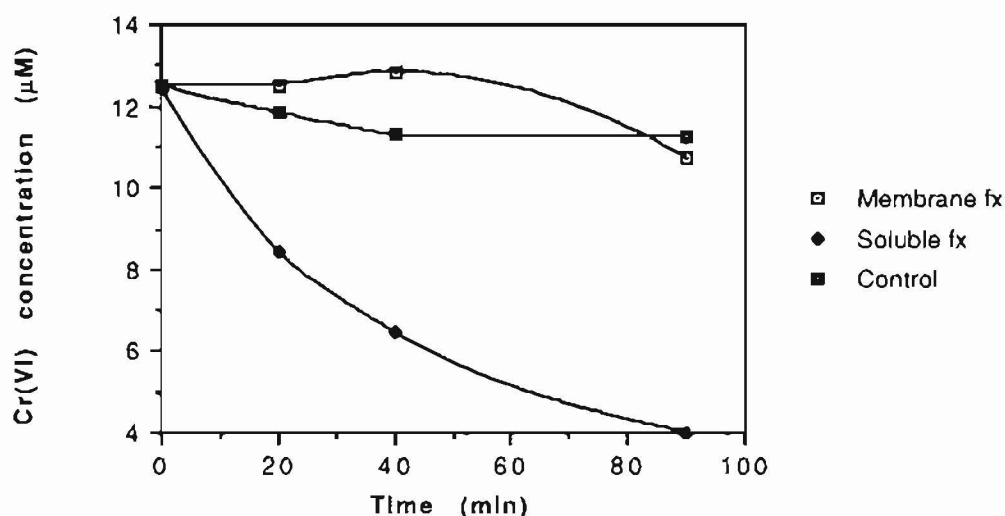


Figure 16. Soluble and membrane fractions(fx) were assayed for Cr(VI) activity in the presence of  $10\mu\text{M}$  NADPH.

## 2. Determination of Reductase Cofactor Dependence

In order to understand the metabolic requirements of the reductase enzyme, a series of coenzyme *in vitro* tests were run on the soluble fraction, where the enzyme was found to reside. In order to illustrate clearly the differential coenzyme activity, data were adjusted so that base line reduction (in the absence of coenzyme) was equal to zero. All other data were adjusted to accommodate this baseline. Thus, Cr(VI) reduction by the enzyme fraction was a function of coenzyme dependence. Figure 17 illustrates data recorded during Cr(VI) reduction in the presence of  $10\mu\text{M}$  NADH.

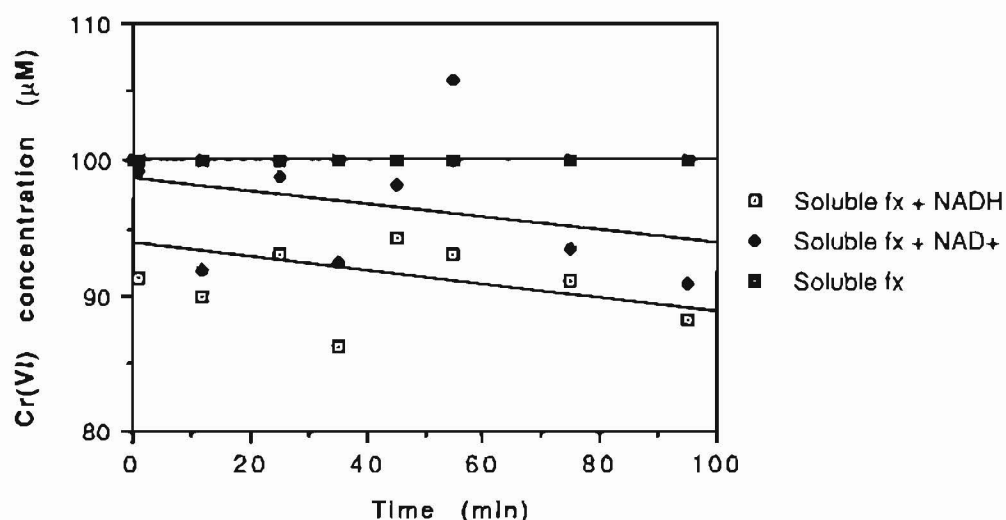


Figure 17. NADH dependence of extracted Cr(VI) reductase. The soluble fraction (fx) was treated with 10  $\mu$ M NADH or NAD<sup>+</sup> and assayed for Cr(VI) reduction with respect to base line reduction in the absence of coenzyme. Cr(VI) reduction in the absence of coenzyme was assigned zero. All data were adjusted to this baseline.

*In vitro* reduction was increased approximately 10% by the presence of NADH, although, given the associated standard error, this observation was rendered suspect. Similarly, *in vitro* protein analysis was carried out in the presence of NADPH. The following data revealed that indeed, Cr(VI) reduction was increased approximately 25% by the presence of this coenzyme.

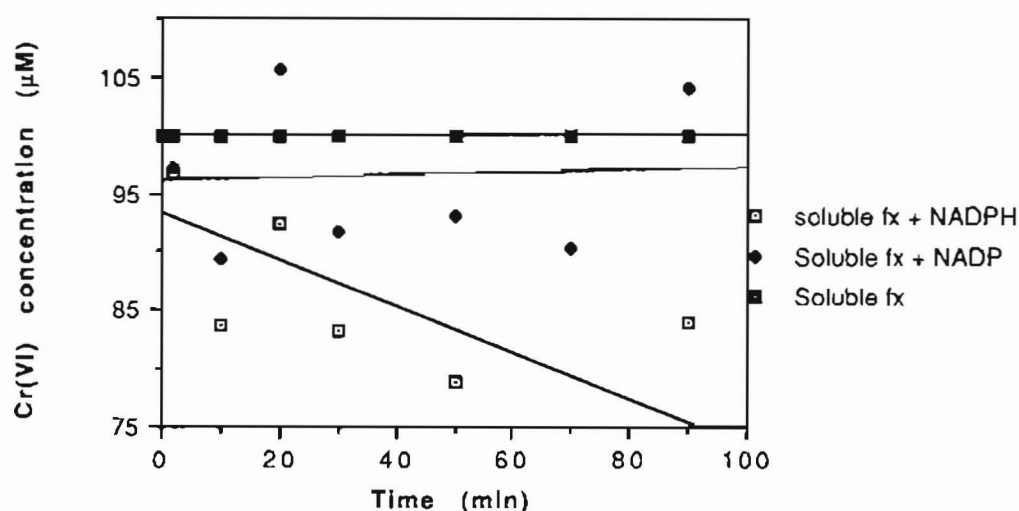


Figure 18. NADPH dependence of extracted Cr(VI) reductase. The soluble fraction was treated with 10  $\mu$ M NADPH or NADP and assayed for Cr(VI) reduction with respect to base line reduction in the absence of coenzyme. As above, Cr(VI) reduction in the absence of coenzyme was assigned zero and all data were adjusted to this baseline.

Finally, the two coenzyme experiments were compared against each other. This was done by assigning a standard baseline reduction for both assays and then adjusting the data for the new parameters relative to that baseline. Figure 19 shows that Cr(VI) reduction in the presence of NADPH occurred at a rate approximately 23% greater than that in the presence NADH. Hence, NADPH is the predominate electron donor for this Cr(VI) enzyme catalyzed reduction.

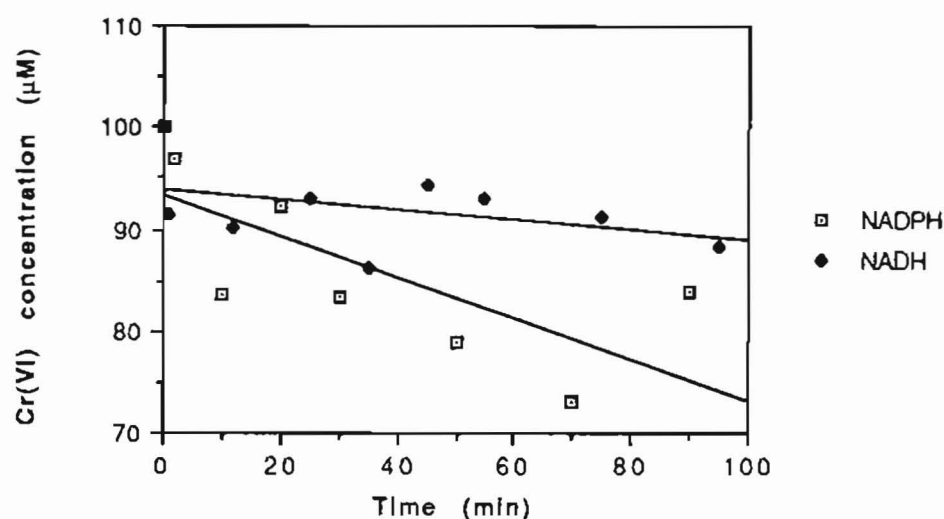


Figure 19. NADH vs. NADPH Cr(VI) reductase dependence. The previous two data sets were compared with respect to a common base line.

### III. GENETIC ANALYSIS

#### 1. Plasmid Curing:

On the assumption that the reductase gene exists on plasmid DNA based upon previous studies of similar genetic traits(1,2,5,6), plasmid curing was attempted. Results were inconclusive because the cells did not lose Cr(VI) reduction capability. Colonies either grew to abundant Cr(VI) reducing populations at very low level ethidium bromide or not at all at higher concentrations of ethidium bromide. These data indicate that the Cr(VI) reductase gene may not have been located on a plasmid. However, negative data of this sort is inconclusive without other experimental verification. It was quite possible that the protocol was ineffective. Hence, a plasmid isolation was performed.

## 2. Plasmid Isolation:

Given the inconclusive data derived from plasmid curing, a plasmid purification was carried out to isolate plasmids contained within the cell. No plasmids were isolated from this isolate using this technique.

In order to prove the effectiveness of the protocol, plasmid DNA purified from an *E. coli* known to contain three plasmids was run with plasmid DNA isolated from *Staphylococcus sp.* using the identical protocol. Hoechst stain was employed to assure cell lysis of both bacterial species. Based on these data, complete cell lysis was assured. Figure 20 shows the absence of plasmid DNA native to the *Staphylococcus* strain.



Figure 20. Isolated Plasmid DNA run on 0.8% agarose gel. (A) Lambda phage DNA (digested with Eco RI) sizing standard, (B) *Staphylococcus sp.* plasmid DNA isolation aliquot, and (C) *E. coli* plasmid DNA isolation aliquot were separated and compared.

### 3. Genome Isolation:

Given that the reductase gene may not reside on a plasmid, a genomic library could be established in order to isolate the reductase gene. The first step was isolation of clonable fragments (~15Kb or larger) of genomic DNA. From this isolated genome a genomic library may be established relatively easily. The following gel revealed the clonable genomic DNA isolated from the *Staphylococcus*. A relatively intact genome was isolated and is of sufficient size to allow for cloning protocols.



Figure 21. *Staphylococcus* sp. genomic isolate (B) was separated on a 0.8% agarose gel with Lambda phage DNA (digested with Eco RI) molecular weight standard (A).

## DISCUSSION

Our *Staphylococcus* sp. isolate represents a tool which may be used to understand the physiology of Cr(VI) reduction, and may also be used in bioremediation applications. Although this organism's Cr(VI) reduction capacity has only been preliminarily characterized, we have attained an understanding of reductase enzyme kinetics, and have also taken the first steps to characterize the reductase gene.

Although Gram positive and Gram negative bacteria are both ubiquitous in nature, Duxbury and Bicknell (5) suggested that Gram negative bacteria were better able to endure toxic metal stress than Gram positive bacteria because of their cell structure.

*Staphylococcus* has rarely been isolated from toxic environmental samples. The most heavily-studied *Staphylococcus* species are pathogens and are responsible for many human diseases including pneumonia, meningitis, osteomyelitis, scalded skin syndrome, impetigo, and toxic shock syndrome among others.(8)

Given the statistics provided by fatty acid analysis it is difficult to conclude that these bacteria were *Staphylococcus* sp. Although this organism's fatty acid composition closely resembled *Staphylococcus*, it was hardly a match. It has been estimated that only 1% of all bacterial species have been identified. Certainly, this isolate may be a close relative of *Staphylococcus*. However, it could very well be a previously unidentified organism.

Aside from morphological similarities not discussed in this paper, identification of the isolate as a *Staphylococcus* species was



given validity by the organism's resistance to lysis. After treatment with sonication, freeze-thaw, lysozyme, and freeze-thaw/lysozyme the bacteria remained intact and functional. This resilience under stress is a distinct characteristic of *Staphylococcus*. Due to a very thick cell wall composed of peptidoglycan crosslinked by pentaglycine bridges the bacterium resists environmental osmotic stress (24).

Lysostaphin specifically lyses *Staphylococcus* cell wall by hydrolyzing the pentaglycine bridges. These bridges are found in much higher concentrations in *Staphylococcus* than other cell types and are responsible, in large part, for the cell's rigidity. Lysostaphin is specific to *Staphylococcus*. It has no effect on other genera (20). Therefore, the fact that lysis occurred in the presence of this enzyme further corroborated the isolate's identity.

Perhaps as a result of thick cell walls and rigidity, this isolate has a competitive edge over sporulating bacteria due to its ability to withstand very high hypertonic levels. *Staphylococcus sp.* was able to withstand the very high salt concentrations found in the Sandia dump site. This is also a *Staphylococcus* characteristic (8). Thus, this nonsporulating bacterium from Sandia may have been adapted to out-compete other resilient bacteria.

The bacterium may have evolved many mechanisms to compete in this nutrient starved environment as well. By metabolically adapting to environmental sources of oxidizable substrate the isolate conserved energy and allowed for optimal efficiency under a variety of nutrient conditions. Doelman and Haanstra (4) found that the addition of lead to sandy soil retarded

the decomposition of various substrates by *E. coli*, particularly cellulose and millipede excrement. As illustrated by Figure 13, *Staphylococcus* sp. growth on a variety of substrates was inhibited in the presence of Cr(VI). Perhaps this was a similar retardation effect as that observed by Doelman and Haanstra. Alternatively, metabolic pathways may have been inactivated as a result of the cells natural habitat, i.e. the Sandia ecosystem.

Glucose, a common oxidizable substrate, allowed for little growth, and none when tested under 0.5 mM Cr(VI) conditions. Sucrose, however, was a suitable substrate which provided for limited growth under these starvation conditions. Sucrose may be trans-carboxylated and phosphorylated to fructose-6-phosphate, an intermediate of glycolysis which is subsequently oxidized and its products shuttled into the citric acid cycle(24). Hence, a glycolysis bypass may lie early in glycolysis, before fructose-6-phosphate is introduced. The most likely candidate for enzymatic dysfunction was hexokinase, which would have allowed for glucose phosphorylation and entrance into a variety of glycolytic cycles. Together, an abbreviated glycolytic cycle and the citric acid cycle allowed for energy derivation from a variety of oxidizable substrate.

Citric acid cycle function was verified by positive growth in the presence of citrate as well as that recorded in the presence of sucrose. All of the citrate added has the potential to be fully oxidized via the citric acid cycle. However, only half of the energy stored within a sucrose molecule has the potential to be metabolized by cellular processes. Sucrose, a disaccharide, breaks down to fructose and glucose(24). If glucose may not be oxidized by the cell, only half

of the energy tied up in sucrose was used in the form of fructose. Thus, citrate allowed for more efficient growth under nutrient starvation conditions.

The Sandia National Laboratory dumped heavy concentrations of citrate onto the desert sands in addition to a myriad of heavy metals in an attempt to prevent wide spread pollution by these metals. By complexing the metals, citrate prevented their migration through the soil and into the ground water. The isolate, which thrived upon this citrate, may have developed adaptations in order to capitalize upon available substrate. The citric acid cycle remains functional. Simultaneously *Staphylococcus* sp. could have conserved energy by halting production of enzymes for glucose oxidation, thereby inhibiting glycolytic metabolism.

Just as the isolate appeared metabolically adapted to concentrations of oxidizable substrate, it may have become adapted to the desert climate so that it proliferates within a very narrow temperature range around 37°C. This is within the accepted standard temperature range of *Staphylococcus*.

Optimal pH for bacterial proliferation was also determined. It was difficult to compare the environmental conditions from which the bacteria was isolated with laboratory conditions. Although pH studies were never carried out on the desert sands, it might be intuitively reasoned that acidic conditions prevailed as a result of the tremendous amount of citrate present. However, the citrate was dumped into a previously alkaline environment. Therefore, it is difficult to reason environmental pH and thereby compare the experimental value with native conditions. The bacteria was found

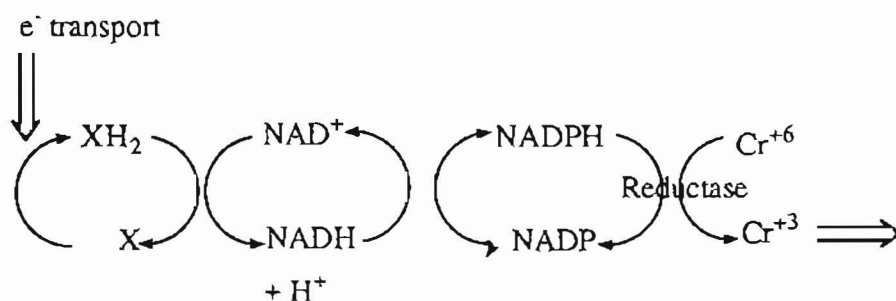
in the laboratory to proliferate most efficiently under basic conditions (pH 8-9).

It is important to recognize that in determining optimal Cr(VI) reduction parameters and the effects of Cr(VI) on microbial mediated processes, samples had to be removed from field sites and transported to the laboratory. Sample manipulation of this sort is a short coming of environmental microbiology. Physiochemical characteristics of any environment influence the chemical form and mobility of heavy metals and hence their ability to interact with microorganisms (4). The laboratory sample conditions were not indicative of the environment from which the isolate was drawn. Hence, results derived from this line of study were slightly biased by experimental parameters. Any potential bioremediation application using these data would need to be accompanied by an integral *in situ* microbial ecology study.

The isolate, found to tolerate and reduce up to 3 mM Cr(VI), represents a potentially powerful tool for Cr(VI) detoxification. The knowledge of optimal Cr(VI) reduction parameters may allow for bioremediation applications. This type of bioremediation application was seen in Prince William Sound, Alaska in the wake of the Exxon Valdez accident. Indigenous bacteria capable of digesting hydrocarbons were given nitrogen, phosphorus, and potassium supplements to augment growth. Oil degradation on the shores occurred at a rate faster than the natural rate (10). This Alaskan test case is analogous to the case of these *Staphylococcus* in bioremediation applications

Although these optimal physiological parameters were essential for any environmental applications of this bacterium relative to its Cr(VI) reduction capacity, protein and genetic analysis were equally important in determining reductase function and development of auxiliary bioremediation schemes. The ability to manipulate the reductase function, as opposed to simply enhancing it, will allow for a broader range of applications. These applications range from accelerated cleanup of contaminated ground water to detoxification of landfills.

Protein analysis yielded a Cr(VI) reductase protein which resides in the cytosol. NADH enhanced *in vitro* activity by approximately 10%, and NADPH by 30%. This leads to the conclusion that NADPH was the primary reducing agent, and that NADH played an auxiliary role. The following mechanism is proposed to account for this auxiliary role of NADH:



NADPH was oxidized to NADP allowing the reductase mediated reduction of Cr(VI) to Cr(III). The oxidized NADP then underwent enzyme mediated reduction by NADH which was thereby oxidized to  $NAD^+$ . The  $NADH$  could then be regenerated by normal cellular processes.

This proposed mechanism accounts for the enhanced Cr(VI) reduction in the presence of NADPH, and for less enhanced reduction in the presence of NADH. As NADH acts primarily to reduce NADPH its effects were limited by the concentration of NADPH *in vivo*. *In vitro* we found that NADH also may act as a reducing agent, but this may be the result of direct NADH reduction of Cr(VI), and not reductase mediated (6). A similar, and actual redox scheme for iron reductases lends validity to this hypothesized mechanism(16).

Using this method, it was unclear whether Cr(VI) was reduced directly to Cr(III), or if there were intermediate steps. Suzuki (19) has postulated a Cr(VI) mechanism for *Pseudomonas* involving Cr(V) intermediates. Also, coenzyme and enzyme kinetics remain unknown. A great deal more study is required to elucidate the specific reduction mechanism relative to the physiology and kinetics associated with it.

It is also important to recognize that the reductase function is not necessarily linked to Cr(VI) tolerance. Cr(VI) reduction is probably a side effect of an enzyme which has other, as of yet unidentified, natural substrates. Cr(VI) tolerance is probably mediated by a separate and distinct mechanism. (20)

Having isolated the reductase fraction, purification of the protein from the fraction was planned, but was not possible due to time constraints. A purified protein would be highly active, although possibly less stable as well. This high activity would allow for accurate enzyme studies; without the purified protein, enzyme kinetic studies are difficult.

More than protein isolation, gene localization represents a powerful technique for confirming reductase function and for potential bioremediation applications. The ability to manipulate the reductase gene will allow for more comprehensive study and understanding.

The data seem to indicate that the bacteria contained no plasmid DNA and that the reductase gene therefore resided in the genome. It is important to recognize that negative data of this sort are very difficult to verify. It was quite possible that low copy plasmid DNA escaped detection. However, exclusive genome control might be an adaptive advantage for the cell. Plasmids are often lost or diluted from cell populations. As this gene is essential for the organism's survival in the Sandia chromate dump, loss of this gene would prove costly.

Transformation, conjugation, and transduction are generally accepted mechanisms of gene transfer among bacteria in laboratory cultures. The transfer of drug resistance mechanisms and the adaptation of microbial communities to the degradation of pesticides and other unusual compounds have provided evidence that similar plasmid exchanges occur in nature. As many bacterial metal tolerance mechanisms are encoded by plasmid borne genes (6), it seems possible that, over time, a plasmid with a reductase gene could have become incorporated into the genome. If the isolate does not expend any energy in plasmid maintenance, it may therefore have developed a evolutionary selective advantage over many others in this toxic environment.

In order to isolate the reductase gene, all cellular genetic material was isolated. This method would allow for reductase gene isolation regardless of its location (plasmid or genome). A genomic library may be subsequently established. Again, time constraints have disallowed this. A genomic library is established by cutting the genomic DNA into many pieces with restriction enzymes. The genomic DNA fragments isolated were well above that size (close to 100Kb) and make good candidates for inclusion in a library (??). These various pieces of the genome may then be incorporated into engineered plasmids and transformed into another cell line which does not have the reduction capability. These clones would then be screened for Cr(VI) tolerance and reduction.

The ability to manipulate the Cr(VI) gene has potentially useful applications. At the Prince William Sound disaster bioengineered bacteria were also released into the oceans in an effort to detoxify that media. Due to insufficient ecological study, however, the bacteria quickly crashed under difficult conditions in the Arctic ocean. This introduction of bioengineered bacteria was a test case which is likely to become more applicable, despite its initial failure.

An ability to manipulate this Cr(VI) reductase gene may allow for the engineering of various other bacteria so that Cr(VI) reduction may be employed under any given set of environmental parameters. This application is limited, however, by government legislation which prohibits the introduction of nonindigenous life to established ecosystems(10). With further study, however, there is the probability that this regulation will be modified to allow for this type of bioremediation application under extreme conditions such as that



at Prince William Sound or at the Sandia National Laboratory dump site.

Another potential bioremediation application combines an understanding of protein kinetics with genetic manipulation. The isolated reductase gene may be recombined into an operon, such as the iron siderophore operon which has an overactive promoter in the presence of iron which will thus produce bulk quantities of the protein for detoxification applications. A purified protein may be potentially applied directly to affected sites or used in bioreactors (10). Stimulated by the proper conditions (i.e., coenzyme, pH) the protein will perform the reduction capacity before denaturing.

In addition to the bioremediation applications made available by bioengineering, a great deal of evolutionary knowledge may also be derived from gene localization. It may allow for an evolutionary comparison study between Cr(VI) reducing species. This *Staphylococcus* sp. appears to be the first documented bacteria of the genus (assuming that it is *Staphylococcus*) with the Cr(VI) reducing ability. It would be interesting to ascertain the evolutionary relevance of this Cr(VI) reductase from this strain.

In conclusion, we have derived a fairly stringent set of parameters to allow for optimal Cr(VI) reduction. These experimental findings may complement ecological studies so that these bacteria may bioremediate the toxic Sandia dump. In addition, we have made advances in characterizing the genetic and protein profile for this reductase function. Much remains to be accomplished in order to attain a complete understanding of this remarkable *Staphylococcus* trait.

## BIBLIOGRAPHY

1. Brock T. and M. Madigan. 1991. Biology of Microorganisms. Prentice Hall, Englewood Cliffs, NJ.
2. Broda, P. 1979. Plasmids. WH Freeman, San Fransisco.
3. Das S. and A.L. Chandra. 1989. Chromate reduction in *Streptomyces*. *Experimentia* 46:731-733
4. Doelman, P., and Haanstra. 1979. Effects of Lead on the Decomposition of Organic Matter. *Soil Biol Biochem* 11: 481-485
5. Duxbury, T., and Bicknell, B. 1983. Metal Tolerant Organisms from Natural and Metal Polluted Soils. *Soil Biol. Biochem* 15: 243-250
6. Duxbury, T. 1988. Ecological Aspects of Heavy Metal Responses in Microorganisms, p.185-229 in KC Marshall (ed.) *Advances in Microbial Ecology*. Plenum Press, NY.
7. Dyer, D.W., Rock, M.I., and Lee, C.Y. 1985. Generation of transducing particles in *Staphylococcus aureus*. *Journal of Bacteriology*. 61: 91-5
8. Elek, S.D. 1959. *Staphylococcus Pyogenes* and its Relation to Disease. Edinbergh, Livingstone.
9. Fekete F.A. and L.L Barton. 1991. Effects of iron(III) analogues on growth and pseudomonas synthesis in a chromium-tolerant *Pseudomonas* isolate. *Biol Metals* 4:211-216
10. Fekete, F.A. 1994. Bioremediation of Aqueous and Soil Environments Contaminated with Toxic Wastes. In Press. University of Akron Press.
11. Friberg, L. (ed.) 1986. Handbook on the Toxicology of Metals. Elsevier, Amsterdam.

12. Gerhardt P., R.G.E. Murray, W.A. Wood, N.R. Krieg. (ed.). 1994. Methods for General and Molecular Bacteriology. ASM, Washington D.C.
13. Harte, J. 1991. Toxics A to Z. Berkley. UCAL press.
14. Horitsu H., S. Futo, Y. Miyazawa, S. Ogai, and K. Kawai. 1987. Enzymatic Reduction of Hexavalent Chromium by Hexavalent Chromium Tolerant *Pseudomonas ambigua* G-1. Agric Biol Chem 51:2417-2420
15. Ishibashi Y., C. Cervantes, and S. Silver. 1990. Chromium Reduction in *Pseudomonas putida*. Appl Environ Microbiol 56:2268-2270
16. Lodge, L. 1993. Enzymatic Reduction of Iron in Siderophores, p241-267 in Barton L.L. and B.C. Hemming (ed.), Iron Chelation in Plants and Soil Microorganisms. Academic Press. Boston,.
17. Lovley D.R., E.J.P. Phillips 1994. Reduction of Chromate by *Desulfovibrio vulgaris* and Its  $c_3$  Cytochrome. Appl Environ Microbiol 60: 726-728
18. Mandelstam J., McQuillen K., Dawes I. (ed.) 1982. Biochemistry of Bacterial Growth. Wiley and Sons. New York.
19. Mikalsen A, J. Alexander, H. Wallin, M. Ingelman-Sunderberg, and R.A Andersen. 1991. Reductive metabolism and protein binding of chromium(VI) by P450 protein enzymes. Carcinogenesis 12:825-831
20. Ornston L.N., A. Balows, and E.P Greenberg (ed.) 1993. Annual Review of Microbiology. Palo Alto, CA.
21. Suzuki T., N. Miyata, H. Horitsu, K. Kawai, T. Takamazawa, Y. Tai, and M. Okazaki. (1992). NAD(P)H-Dependent Chromium(VI) Reductase of *Pseudomonas ambigua* G-1: a Cr(V) Intermediate Is Formed during the Reduction of Cr(VI) to Cr(III). Journal of Bacteriology 174:5340-5345
22. Tuncan E.U. and S.E. Martin 1986. Lysostaphin Lysis Procedure for Detection of *Staphylococcus aureus* by the Firefly Bioluminescent ATP Method. Appl Environ Microbiol 53:88-91